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(54) Title: REGULATION OF T CELL–MEDIATED IMMUNITY BY TRYPTOPHAN

(57) Abstract

A mechanism of macrophage–induced T cell suppression is the selective elimination of tryptophan and/or increase in one or more tryptophan metabolites within the local macrophage microenvironment. Studies demonstrate that expression of IDO can serve as a marker of suppression of T cell activation, and may play a significant role in allogeneic pregnancy and therefore other types of transplantation, and that inhibitors of IDO can be used to activate T cells and therefore enhance T cell activation when the T cells are suppressed by pregnancy, malignancy or a virus such as HIV. Inhibiting tryptophan degradation (and thereby increasing tryptophan concentration while decreasing tryptophan metabolite concentration), or supplementing tryptophan concentration, can therefore be used in addition to, or in place of, inhibitors of IDO. Similarly, increasing tryptophan degradation (thereby, decreasing tryptophan concentration and increasing tryptophan metabolite concentration), for example, by increasing IDO concentration or IDO activity, can suppress T cells. Although described particularly with reference to IDO regulation, one can instead manipulate local tryptophan concentrations, and/or modulate the activity of the high affinity tryptophan transporter, and/or administer other tryptophan degrading enzymes. Regulation can be further manipulated using cytokines such as macrophage colony stimulating factor, interferon gamma, alone or in combination with antigen or other cytokines.

5   **REGULATION OF T CELL-MEDIATED IMMUNITY BY
TRYPTOPHAN**

The United States government has certain rights in this invention by
virtue of National Institutes of Health K08 grant to David Munn.

10   This application claims priority to U.S. Serial No. 60/067,610 entitled
"Regulation of T Cell Activation" filed December 5, 1997; U.S. Serial No.
60/080,384 entitled "Regulation of Pregnancy" filed April 1, 1998; and U.S.
Serial No. 60/080,380 entitled "IDO Inhibitors for Use as Antiviral Agents"
filed April 1, 1998, by David Munn and Andrew Mellor.

15   The present invention is generally in the area of regulation of T cell
activation using modulators of the enzyme indoleamine 2,3-dioxygenase
(IDO) which is used by immunosuppressive antigen-presenting cells such as
tissue macrophages and placental trophoblasts to prevent T cells from
activating in response to antigens presented by these cells. Modulation of the

20   enzyme activity can therefore be used to affect pregnancy, infection by
certain viruses such as HIV, and inflammation. More specifically, the
present invention includes altering maternal tolerance of pregnancy using
modulators of the enzyme indoleamine 2,3-dioxygenase (IDO) which is used
by immunosuppressive antigen-presenting cells such as tissue macrophages

25   and placental trophoblasts to prevent T cells from activating in response to
antigens presented by these cells.

Background of the Invention

Traditionally, the "professional" antigen-presenting cells (APCs) of
the myeloid lineage, dendritic cells and macrophages, have been viewed

30   primarily as accessory cells, functioning simply to assist T cell activation.
Recently, however, it has become clear that myeloid-lineage APCs exert a
profound influence on T cells, regulating both the nature of the response
(humoral versus cellular immunity) and, in some cases, even whether a
response occurs at all (activation versus anergy). This has been the subject

35   of recent reviews by Fearon and Locksley (*Science* 1996;272:50-54) and
Trinchieri and Gerosa (*J. Leukocyte Biol.* 1996;59:505-511). Currently, the

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5   biology of myeloid-lineage APCs is not well understood. Dendritic cells and

     macrophages appear to derive from a common progenitor in the

     myelomonocytic lineage (Szabolcs, et al. *Blood* 1996;87:4520-4530), but

     their markedly different functional characteristics are determined during a

     complex process of hematopoietic differentiation, which continues well after

10   their exit from the bone marrow (Thomas, et al. *Stem Cells* 1996;14:196-

     206). Hematopoietic differentiation has traditionally fallen outside the

     purview of classical immunology.

            Macrophages enter the tissues at the immature stage of circulating

     monocytes. Using *in vitro* models, it has been shown that the cytokine

15   milieu which they encounter at this early stage determines the phenotype

     which they will subsequently adopt. Under the influence of certain cytokines

     (in humans, usually GM-CFS plus IL-4 or TNF), monocytes differentiate *in*

     vitro into cells which closely resemble dendritic cells (Mackensen, et al.

     Blood 1995;86:2699-2707; Rosenzwajg, et al., *Blood* 1996;87:535-544). In

20   the presence of inflammatory cytokines they differentiate into macrophages

     activated for antigen presentation and host defense (Munn, *Cancer Res.*

     1993;53:260-2613; Morahan, et al. In: Heppner GA, Fulton AM, eds.

     Macrophages and Cancer. Boca Raton, FL;CRC Press, 1988:1-16.). In the

     absence of inflammatory cytokines, monocytes differentiate under the

25   influence of their lineage-specific growth factor, MCSF, into a type of

     macrophage which inhibits, rather than supports, T cell activation (Munn, et

     al. *J. Immunol.* 1996;156:523-532).

            The adaptive immune system must tailor the T cell repertoire so as

     not to respond to self antigens. The classical model (reviewed by Nossal in

30   *Cell* 1994;76:229-239) holds that autoreactive T cell clones are deleted in the

     thymus via the process of negative selection (encounter with antigen at the

     immature thymocyte stage triggers apoptosis, resulting in clonal deletion).

     Although the thymus undoubtedly provides a major site of negative

     selection, there are two difficulties with this model. First, it would seem

35   unlikely that every developing T cell could be exposed to every self protein

     during its relatively brief transit through the thymus. Second, autoreactive T

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5   cells are empirically found in the peripheral blood of normal, healthy hosts
     (Steinman *Cell* 1995;80:7-10). This suggests that there must exist some
     additional means of tailoring the T cell repertoire after the T cells have left
     the thymus, a process now designated peripheral tolerance. Multiple
     mechanisms have been proposed to contribute to peripheral tolerance (for

10   recent reviews see Steinman L.*Cell* 1995;80:7-10; Mondino, et al. *Proc.
     Natl. Acad. Sci USA* 1996;93:2245-2252; and Quill H. *J. Immunol.*
     1996;156:1325-1327).

         Most recent studies support a model in which dendritic cells are the
     primary physiologic route of antigen presentation to T cells (Thomas 1996).

15   Under normal circumstances, this process is felt to occur only in lymph
     nodes. Given this exclusive role for dendritic cells in initiating immune
     responses, tissue macrophages represent a paradox. They are professional
     APCs, but they are also professional scavengers of all manner of damaged
     cells and proteins, and hence take up a huge array of self antigens.

20   Moreover, unlike dendritic cells, many of them constitutively express MHC
     and costimulatory ligands (Azuma, et al. *Nature* 1993;366:76-79) and
     function as APCs *in vitro* (Unanue, et al. *Science* 1987;236:551-557),
     implying they are constantly prepared to present antigen.

         It is not known how they avoid provoking autoimmune responses.

25   One possibility is that T cells never encounter tissue macrophages. This may
     indeed be the case for naive T cells, since they are not thought to circulate
     through tissues (Springer, et al. *Cell* 1994;76:301-314). However, at times
     of injury and inflammation, many self antigens unavoidably enter the normal
     antigen-presentation pathway along with legitimate foreign antigens (either

30   because the dendritic cell has no way to discriminate between the two, or due
     to influx of debris from damaged tissues into the draining lymph nodes
     (Steinman1995)).

         Certain pathological conditions, such as AIDs (caused by the human
     immunodeficiency virus, HIV) and latent cytomegaloviral (CMV) infections,

35   are extremely difficult to treat since the macrophages act as reservoirs for the
     viruses. Even though the cells are infected with virus, they are not

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5   recognized as foreign. It is not known why these cells are protected from the host's immune system.

It is therefore an object of the present invention to identify mechanisms by which tissue macrophages regulate T cell activation in order to modulate autoimmune responses to the self-derived antigens which they

10   present, especially in the context of infections with facultative intracellular pathogens, such as HIV and CMV.

It has long been a mystery why a pregnant individual does not reject her fetus as foreign. Many theories have been proposed, and various mechanisms suggested. Being able to understand and control this

15   phenomena would be of benefit both for the development of contraceptives or aborticides, as well as in treatment of some women who are unable to carry a fetus full term. Medawar, 1953, *Symp. Soc. Exp. Biol.* 7, 320-328, pointed out 45 years ago that the mammalian conceptus ought to survive gestation because it carries and expresses paternally-inherited polymorphic

20   genes that provide maternal immune responses when expressed by other tissues. The paradox presented by survival of fetal allografts has not yet been explained in mechanistic terms despite much research on the immunology of mammalian reproduction.

Three factors that might explain the immunological paradox of fetal

25   survival are: (1) anatomic separation of mother and fetus, (2) antigenic immaturity of the fetus and (3) immunologic "inertness" (tolerance) of the mother (Medawar 1953). Recently, attention has focused on the third possibility based on evidence that the entire maternal T cell repertoire is transiently tolerized to paternal MHC class I alloantigens during pregnancy

30   (Tafuri, et al., 1995, Science 270, 630-633). However, it is not clear how transient tolerance is imposed and maintained in the peripheral T cell repertoire during pregnancy.

It is therefor an object of the present invention to identify mechanisms by which rejection of the fetus by its mother are prevented.

35   It is a further object of the present invention to provide reagents and methods for use thereof for terminating or maintaining pregnancies.

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Summary of the Invention

A mechanism of macrophage-induced T cell suppression is the selective elimination of tryptophan and/or increase in one or more tryptophan metabolites within the local macrophage microenvironment via simultaneous

10   induction of the enzyme indoleamine 2,3-dioxygenase (IDO) and a tryptophan-selective transport system. Studies demonstrate that expression of IDO can serve as a marker of suppression of T cell activation, and may play a significant role in allogeneic pregnancy and therefore other types of transplantation, and that inhibitors of IDO can be used to activate T cells and

15   therefore enhance T cell activation when the T cells are suppressed by malignancy or a virus such as HIV. Inhibiting tryptophan degradation (and thereby increasing tryptophan concentration while decreasing tryptophan metabolite concentration), or supplementing tryptophan concentration, can therefore be used in addition to, or in place of, inhibitors of IDO. Similarly,

20   increasing tryptophan degradation (thereby , decreasing tryptophan concentration and increasing tryptophan metabolite concentration), for example, by increasing IDO concentration or IDO activity, can suppress T cells. Although described particularly with reference to IDO regulation, one can instead manipulate local tryptophan concentrations, and/or modulate the

25   activity of the high affinity tryptophan transporter, and/or administer other tryptophan degrading enzymes. Regulation can be further manipulated using cytokines such as macrophage colony stimulating factor, interferon gamma, alone or in combination with antigen or other cytokines.

Studies demonstrate that expression of IDO can serve as a marker of

30   suppression of T cell activation, and plays a significant role in allogeneic pregnancy. Inhibitors of IDO can be used to activate T cells and thereby induce rejection of a fetus. Studies show that administration of an inhibitor of IDO, 1-methyl-tryptophan, induces specific and uniform rejection of allogeneic conceptus. Embryo loss is preceded by extensive inflammation,

35   the appearance of monomuclear and neutrophil infiltrates, and degeneration of decidual tissues. Rejection is T cell driven since a single paternally-

5   inherited fetal MHC class I alloantigen provokes embryo loss, and rejection
       does not occur if maternal lymphocytes are absent when IDO activity is
       inhibited or the mother does not have functional T cells.

            Administration of an inhibitor of IDO, 1-methyltryptophan, can cause
       rejection of foreign tissue without toxicity. These studies provide a

10   mechanism whereby facultative intracellular pathogens are able to avoid
       destruction by the host's T cells, even when the macrophages express viral
       antigens on their surfaces. Inhibition of IDO should cause the host to attack
       and kill the infected macrophages. In a preferred embodiment for treatment
       of HIV, the viral load is decreased using standard HIV therapy, usually over

15   a period of three to six months. This is then followed by treatment with an
       inhibitor of IDO such as 1-methyltryptophan at a dose equivalent to 1 g/kg,
       for a period of between one day and a few weeks. For treatment of CMV
       infections, especially before cancer chemotherapy or bone marrow
       transplant, the patient is treated with an inhibitor of IDO for a period

20   effective for the patient's own T cells to attack and kill any infected
       macrophages. Malignancies are treated with an IDO inhibitor until the
       tumor(s) is necrotic or the cancer is in remission.

                            Brief Description of the Drawings

25   Figure 1 is a graph of thymidine incorporation (cpm) by T cells in the
       presence of supplemental amino acids. Co-culture conditioned medium is
       selectively depleted of tryptophan. Human monocytes were allowed to
       differentiate into macrophages then co-cultured with mitogen-activated T
       cells (anti-CD3). Fresh PBMC were then suspended in this medium and

30   activated with additional anti-CD3. Replicate cultures were supplemented
       with individual amino acids to the concentrations normally found in RPMI-
       1640. Control cultures received fresh medium. Proliferation was measured
       by 4 hr thymidine incorporation after 72 hrs. Error bars show SD.

            Figure 2 is a graph of thymidine incorporation as a function of

35   tryptophan concentration (nM) showing the dose-response relationship to
       tryptophan. Tryptophan was titrated in co-culture conditioned medium as

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5   described in Figure 1., and proliferation of T cells measured at 72 hrs. SD

was less than10% and error bars are omitted for clarity.

Figure 3 is a graph of tryptophan concentration (micromoles) over

time in hours showing the elimination kinetics of tryptophan in co-cultures.

MCSF-derived macrophages were cultured for 24 hrs with autologous T

10   cells, with or without anti-CD3 mitogen. The medium was replaced with

fresh medium, and supernatant from replicate cultures harvested at the times

shown. The concentration of tryptophan was determined by the

formaldehyde/$FeCl_2$ fluorescence assay. Error bars (less than10%) are

omitted for clarity.

15   Figure 4A and 4B are graphs showing inhibition of the IDO enzyme

abrogates suppression. Co-cultures of MCSF-derived Mϕs and T cells with

anti-CD3 mitogen were supplemented either with the IDO inhibitor-1-

methyl-tryptophan (Figure 4A), or with 10x the normal concentration of

tryptophan (Figure 4B). Proliferation was measured after 72 hrs by

20   thymidine incorporation. The level of background proliferation contributed

by macrophages is indicated by the dotted line. In Figure 4B, macrophages

were seeded at both low density (5 x 10^4/well) and high density (2 x

10^5/well). In both parcels, all differences discussed as significant were $p<.01$

by ANOVA and Tukey's.

25   Figure 5 is a graph of tryptophan degradation (% total) versus

interferon concentration (U/ml) showing that CD40-L acts synergistically

with IFNγ to induce IDO activity. MCSF-derived macrophages were

activated for 24 hrs with various concentrations of recombinant IFNγ, in the

presence or absence of recombinant CD40-L trimer. At the end of the

30   activation period the tryptophan remaining in the cultures was assayed as

described for Figure 3. SD was less than10%.

Figure 6 is a graph of tryptophan degradation (%total) over time

(hours) for showing developmental regulation of inducible IDO activity.

Monocytes were allowed to differentiate in either MCSF or GMCSF+IL-4

35   for 5 days, then activated by sequential exposure to CD40-L and IFNγ.

Following activation, the medium was replaced with fresh medium and the

5   rate of tryptophan catabolism measured over 8 hrs. SD was less than 10%.

Figures 7A and 7B are graphs of tryptophan uptake (pmol/10^6 cells)
over time (min) showing inducible transport of tryptophan into macrophages.
Figure 7A shows uptake of [^{3}H] tryptophan (200 nM) was measured for the
times indicated, then cells were washed onto glass fiber filters and assayed

10   by liquid scintillation counting. The graph compares fresh monocytes,
MCSF-derived macrophages (day 7), and MCSF-derived macrophages
activated for the preceding 24 hrs with IFNγ. All assays were performed in
the presence of 140 mM NaCl. Figure 7B shows sodium-dependent and
sodium-independent (140 mM choline chloride) uptake for T cells activated

15   for 72 hrs with anti-CD3, then assayed as in Figure 7A. A logarithmic scale
is used to permit comparison.

Figures 8A and 8B are graphs of tryptophan uptake (cpm) in the
presence of various competing unlabeled amino acids for dendritic cells
(Figure 8A) and MCSF macrophages (Figure 8B) showing sodium-

20   independent transport of tryptophan in MCSF-derived macrophages does not
have the characteristics of system L. Monocytes were allowed to
differentiate for 5 days in the presence of either GMCSF + IL-4 (dendritic
cells, Figure 8A) or MCSF (macrophages, Figures 8B). Sodium-independent
tryptophan transport was assayed as described in Figure 7 in the presence of

25   various competing unlabeled amino acids (8 mM each).

Figure 9 is a graph of percent inhibition versus concentration of
competitor (tryptophan or phenylalanine) showing that the tryptophan
transport system in MCSF-derived macrophages displays a preferential
affinity for tryptophan. Macrophages were cultured for 7 days in MCSF and

30   activated during the last 24 hrs with IFNγ. Uptake of [^{3}H]tryptophan (400
nM) was then assayed in the presence of various concentrations of unlabeled
tryptophan or phenylalanine.

Figure 10 are HPLC analyses showing elimination of autoreactive T
cells *in vivo* is accompanied by tryptophan degradation. Antigen-transgenic

35   mice received autoreactive T cells by adoptive transfer, and then were
assayed for the tryptophan breakdown product kynurinine at a time when the

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5   T cells were being actively eliminated (day 3). Trace 1 shows HPLC

analysis for kynurinine in control serum prior to adoptive transfer. Trace 3

shows control serum spiked with authentic kynurinine (10 µM). Trace 2

shows serum from mice on day 3 after adoptive transfer of T cells. The

asterisk indicates the kynurinine peak.

10   Figure 11 is a graph of autoreactive TcR-transgenic T cells (1×10^7)

over time (days) after adoptive transfer of cells with and without inhibitor.

Detailed Description of the Invention
Roles of IDO and Tryptophan in T Cell Activation

15   As monocytes differentiate into macrophages they can adopt

markedly different phenotypes, depending on the cytokine milieu.

Macrophages may present antigens in an immunosuppressive, rather than

immunostimulatory fashion. Most phenotypes support T cell activation, but

one, produced by exclusive exposure to the cytokine macrophage colony-

20   stimulating factor (MCSF), suppresses T cell activation. It is proposed that

macrophages discriminate which antigens are "self" based on the presence or

absence of inflammation during terminal macrophage differentiation.

Inflammatory cytokines such as IFNγ (Munn, et al. 1993; Munn, et al.

1996), IFNα, and IL-4 act dominantly over MCSF to prevent the

25   development of the inhibitory macrophage phenotype. Thus,

immunosuppressive macrophages should develop only if they differentiated

in normal, uninflamed tissues. In such tissues, the only antigens encountered

should be self-derived, and hence responding T cells would be presumptively

autoreactive. This is conceptually related to proposed models of self/non-

30   self discrimination such as the model described by Janeway *Immunol. Today*

1993;13:11-16, in which antigens in normal tissues are considered self, and

only in settings of inflammation or infection are T cells allowed to activate.

This model has been subsequently reviewed by Ibrahim in *Immunol. Today*

1995;16:181-186.

35   The data and proposed mechanisms described herein complement

these models by suggesting a specific mechanism by which antigens could be

5   presented in normal tissues in an immunosuppressive fashion. The

       mechanism of this suppression is via induction of the enzyme indoleamine

       2,3-dioxygenase (IDO), which selectively degrades the essential amino acid

       tryptophan. In non-hepatic tissues, the enzyme responsible for the initial

       rate-limiting step in tryptophan degradation along the kynurinine pathway is

10   indoleamine-2,3-dioxygenase (IDO) (Taylor, et al. *FASEB J.* 1991;5:2516-

       2522). IDO is a subject of active current interest in the context of infectious

       disease (Pfefferkorn *Proc. Natl. Acad. Sci. USA* 1984;81:908-912; Gupta

       Infect. Immun. 1994;62:2277-2284; Musso; *Blood* 1994;83:1408-1411;

       Koide *Infect. Immun.* 1994;62:948-955; Daubener *Immunol.* 1995;86:79-84;

15   Daubener, et al., *Eur. J. Immunol.* 1996;26:487-492; Carlin, et al. *J. Leuk.*

       Biol. 1989;45:29-34; Nagineni *Infect. Immun.* 1996;64:4188-4196), where it

       is postulated to reduce the intracellular concentration of tryptophan to the

       point that facultative intracellular pathogens are unable to replicate

       (tryptophan is an essential amino acid for all eukaryotic parasites, and for

20   certain simple prokaryotes). Cell lines which have mutations in the IDO

       gene are unable to control the replication of organisms such as toxoplasma

       and chlamydia (Thomas *J. Immunol.* 1993; 150:5529-5534). Conversely,

       pathogen-sensitive cell lines transfected with IDO acquire the ability to

       inhibit proliferation of these organisms (Gupta *Infect Immun.* 1994;62:2277-

25   2284). IDO may be particularly critical in defense against CNS and ocular

       toxoplasmosis, since glial and retinal cells show a direct correlation between

       IDO activity and control of toxoplasma (Daubener *Eur. J. Immunol.*

       1996;26:487-492; Nagineni *Infect. Immun.* 1996;64:4188-4196). IDO may

       also contribute to the pathophysiology of central nervous system infection,

30   autoimmune inflammation, and AIDS dementia, via both local tryptophan

       starvation and by generation of neurotoxic tryptophan metabolites such as

       quinolinate (Venkateshan *Proc. Natl. Acad. Sci. USA* 1996;93:1636-1641;

       Vogelsgang, *J. Rheumatol.* 1996;23:850-855; *Sardar Neurosci. Let.*

       1995;187:9-12; *Alberati-Giani J. Neurochem.* 1996;66:996-1004).

35   IDO is induced by several inflammatory mediators, including

       interferons and LPS, as well as by viral infection (probably indirectly via

5   interferons). The most potent inducer of IDO is IFNγ, due to two interferon-
stimulated response elements (ISREs) in the IDO promoter (Konan J. Biol.
Chem. 1996;271:19140-19145; Chon J. Biol. Chem. 1996;271:17247-
17252). In addition to its effect on intracellular organisms, IDO appears to
mediate at least part of the antiproliferative effect of IFNγ on replicating host
10   cells, such as virally infected cells and tumor cells (Aune, et al., 1989 *J. Clin.
Invest.* 84, 863-875). Mutant cell lines selected for their ability to grow in
the presence of IFNγ were found to have acquired mutations in the IDO gene
or its promoter (Feng Proc. Natl. Acad. Sci USA 1989; 86:7144-7148.).
Allogeneic tumor cells being rejected by the host immune system *in vivo*
15   upregulate IDO, and this effect is mediated by IFNγ (Takikawa, et al. In:
Schwarcz R, ed. Kynurenine and Serotonin Pathways. New York: Plenum
Press, 1991:437-444; Yu, et al., *Intl. Immunol.* 1996;8:855-865), although it
it not known whether IDO plays a causal role in tumor rejection. Thus, IDO
can inhibit proliferation of both intracellular pathogens and host cells.

20   The prior art in the field has viewed IDO solely as a means to reduce
the concentration of tryptophan within the IDO-expressing cell (Taylor and
Feng, 1991 *FASEB J.* 5:2516-2522). There has been no speculation that IDO
could function to suppress proliferation of neighboring cells, and specifically
no suggestion that it could be used to suppress T cell activation. Based on
25   the *in vitro* studies described herein, the hypothesis was formulated that cells
expressing IDO would be able to suppress T cell activation *in vivo*. From
this hypothesis the testable prediction was formulated that inhibition of IDO
enzyme activity with compounds such as 1-methyl-tryptophan would allow
enhanced T activation in vivo. Review of the existing literature suggested
30   that IDO activity was likely to be present in macrophage-like and dendritic-
like cells in organs of the immune system (Moffett, et al., 1994 *Cell Tissue
Res.* 278:461-469). Therefore, to test the hypothesis that IDO-expressing
cells in the immune system suppressed autoreactive T cells in vivo, adoptive
transfer studies were performed in which T cells which were transgenic for a
35   defined T cell receptor were injected into recipient mice made transgenic for
the cognate target antigen. This model of autoimmune T cell activation has

5   been previously described by Tarazona, et al., 1996 *Int. Immunol.* 8:351-358.
       As shown in Figure 10 , the injection of autoreactive T cells resulted in
       induction of IDO (measured as elevated kynurenine in serum). When IDO
       activity was inhibited in recipient animals by administration of 1-methyl-
       tryptophan, the activation of the autoreactive T cells was markedly enhanced
10   (2- to 3-fold over control, as shown by Figure 11). These data thus
       supported the hypothesis that there existed a population of IDO-expressing
       cells which suppressed autoreactive T cells *in vitro*.

       The *in vitro* data presented herein were derived using human
       monocyte-derived macrophages. For convenience, the term "macrophage" is
15   therefore used to describe any IDO-expressing cell which suppresses T cell
       activation. However, *in vivo* this cell could be a macrophage, dendritic cell,
       or other cell type with expresses IDO and suppresses T cells.

       As determined in the studies described herein, macrophages are
       primed during differentiation in the presence of MCSF to undergo a massive
20   induction of IDO activity in response to a synergistic combination of signals
       displayed during early T cell activation (IFNγ plus CD40 ligand). In
       addition, these macrophages markedly increase their rate of tryptophan
       uptake, via induction of a previously unidentified high-affinity transport
       pathway. Macrophages which have differentiated under the influence of
25   macrophage colony-stimulating factor (MCSF) inhibit attempted T cell
       activation via super-induction of the antimicrobial host-defense enzyme
       indoleamine 2,3-dioxygenase, which selectively degrades the essential amino
       acid tryptophan. Simultaneously, these macrophages also markedly increase
       tryptophan uptake via induction of a novel high-affinity transport pathway.
30   The combination of these two mechanisms allows macrophages of this
       phenotype to effectively deplete tryptophan from the local
       microenvironment, thus preventing T cell activation.

       Nutrient depletion is an evolutionarily ancient strategy, and as the
       utility of L-asparaginase against leukemia attests it remains an effective one.
35   The best studied example of this type of mechanism in humans is chelation
       of iron, an essential micronutrient for all dividing cells. Microbes employ

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5   this strategy against each other (deferoxamine is an example), and so do

macrophages via the secretion of the iron-chelating protein lactoferrin

(Baynes *Adv. Exp. Med. Biol.* 1994;357:133-141). Macrophages use iron

depletion as a cytostatic mechanism against tumor cells and other dividing

cells (reviewed in Weiss, et al. *Immunology Today* 1995;16:495-500), and

10   iron is rapidly sequestered from serum and tissues by macrophages in

response to infection and inflammation. Another example of regulation by

nutrient depletion is the ability of macrophages to alter the intracellular thiol

pool in T cells via degradation of cysteine, resulting in thiol-mediated redox

regulation of T cell activation (Gmunder, et al. *Cell. Immunol.*1990;129:32-

15   46; Iwata, et al., *J. Immunol.* 1994;152:5633-5642). Thus, tryptophan

degradation is not a unique phenomenon, but rather belongs to a class of

mechanisms whereby proliferation is inhibited by local depletion of a factor

essential for growth. Like macrophages themselves (Ottaviani, et al.

Immunol. Today 1997;18:169-174), the IDO gene is evolutionarily ancient.

20   An homologous gene exists in lower invertebrates, and the intron-exon

structure has been highly conserved throughout 600 million years of

evolution (Suzuki, et al. *Biochem. Biophys. Acta* 1996;1308:41-48). This

suggests that tryptophan degradation is an effective and important strategy.

A key assumption implicit in the hypothesis is that macrophages *in*

25   *vivo* are able to create a local microenvironment in which the tryptophan

concentration is very low, despite the availability of tryptophan in the

bloodstream. In theory, this should be possible because the rate of

tryptophan delivery is limited by diffusion through the interstitial space.

Burke, et al, have directly tested this assumption by measuring tryptophan

30   concentrations in tumor xenografts which had been induced to express IDO

by IFNγ (Burke, et al. *Int. J. Cancer* 1995;60:115-122). These authors found

an approximately 80% reduction in tumor tryptophan in IFNγ-treated

animals compared to controls, indicating that the rate of local degradation

did indeed exceed the rate of delivery.

35   The IDO enzyme is known from published literature to be expressed

in syncytiotrophoblast cells of the human placenta (Kamimura, et al., 1991

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5   *Acta. Med. Okayama* 45:135-139). Syncytiotrophoblasts are fetal-derived
      cells which comprise the zone of contact between fetal tissues and the
      maternal bloodstream (and hence contact with the maternal immune system).
      The presence of IDO in placenta had been confusing to researchers in the
      field, since the placenta supplies nutrients to the fetus and it seemed

10   paradoxical that it should actively degrade an essential nutrient such as
      tryptophan. Based on the *in vitro* and *in vivo* studies described herein, the
      hypothesis was formulated that the role of IDO in the placenta was to
      suppress T cell responses against the genetically "foreign" fetus. The
      survival of the mammalian fetus has long been a paradox in the field of

15   immunology (Medawar, 1953, *Symp. Soc. Exp. Biol.* 7:320-328), since half
      of the fetus's genes are paternally inherited, and hence should provoke an
      allogeneic rejection response from the maternal immune system. Based on
      the data herein, it was predicted that if IDO activity were inhibited in
      placenta the fetus would be unable to protect itself against maternal T cells,

20   and would be rejected. As shown in Table 1, inhibition of IDO with 1-
      methyl-tryptophan resulted in prompt, T cell-mediated rejection of all
      allogeneic fetuses. There was no effect of inhibitor on genetically identical
      (syngeneic) control fetuses, demonstrating that the inhibitor itself was not
      toxic. This provided definitive support for the ability of IDO to suppress T

25   cell responses *in vivo*. It also showed that IDO-expressing cells other than
      macrophages (in this case, trophoblasts) were able to suppress T cell
      responses, indicating that IDO is a broadly applicable mechanism of immune
      suppression. Specifically, based on the data herein, it is believed that
      antigen-presenting cells (such as macrophages and dendritic cells)

30   throughout the immune system use IDO to suppress unwanted T cell
      responses (e.g., to self antigens).

           The fetus represents a dramatic example of a solid-tissue "allograft"
      which is tolerated by the maternal immune system throughout gestation.
      Prior to the discoveries described herein, it was uncertain whether the fetus

35   presented the transplantation antigens needed to provoke rejection, and, if it
      did, why the maternal immune system chose to tolerate these antigens. The

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5   data in the examples reveal that the fetus does indeed present the relevant

        transplantation antigens (a difference in even a single MHC gene was

        sufficient to provoke rejection when IDO was inhibited, see Table 3), and

        that the maternal immune system did not "choose" to tolerate the fetus, but

        rather was actively prevented from mounting a response by placental

10   expression of IDO. Taken together, these observations lead to two categories

        of clinical applications:

                Category 1: Since IDO and tryptophan catabolism represent a new

        mechanism of T cell suppression, the introduction of the IDO gene by

        transgenesis, the use of pharmacologic inducers of IDO enzyme activity, or

15   the direct introduction of IDO or IDO-like enzymatic activity as purified

        proteins into relevant sites, can all be used for local immunosuppression.

                Category 2: Since inhibition of IDO is able to restore T cell

        responses which would otherwise be suppressed (as in the pregnancy model

        described herein), pharmacologic inhibitors of the IDO enzyme could be

20   used to restore desirable T cell responses which are normally suppressed by

        IDO.

                With regard to Category 1 (use of IDO as an immunosuppressant),

        the most direct application is to simply inject or otherwise deliver the

        purified IDO enzyme as a local immunosuppressive agent. However,

25   because IDO contains a heme prosthetic group and requires a complex

        regenerating system in order to avoid auto-oxidation in vitro (Sono, et al.,

        1980. _J. Biol. Chem._ 255:1339-1345), it may be difficult to employ the

        purified enzyme directly as a pharmacologic agent. A strategy to surmount

        this difficulty is to use purified bacterial enzymes such as indolyl-3-alkane

30   alpha-hydroxylase, which have IDO-like tryptophan-degrading activity and

        which have been shown to function in purified form (U.S. Patent Nos.

        5,723,325 and 5,244,807 to Murtfeldt, et al).

                A second approach is to confer IDO enzymatic activity in a tissue-

        specific manner by the use of IDO as a transgene under an inducible or

35   constitutive promoter. There is currently significant interest in developing

        genetically modified animals (e.g., swine) to serve as donors for solid organ

                                             15

5   xenotransplantation (Saadi and Platt 1998 *Life Sci.* 62:265-287). Based on

the data herein demonstrating the role of IDO in preventing rejection of the

allogeneic fetal "allograft", the use of IDO as a transgene (either as a

germline transgene in genetically modified animals, or delivered by

transfection into human organs) can be applied to xenogeneic and allogeneic

10   human organ transplantation.

Likewise, there exists a body of literature supporting the use of

genetically modified cells to convey immunosuppressive genes into specific

anatomic sites of inflammation, e.g., into the synovial space in arthritic joints

(Jorgensen and Gay 1998. *Immunol. Today* 19;387-391), for purposes of

15   local immunosuppression, or systemically for purposes of inducing tolerance

to subsequent allograft transplantation. These would be additional

applications of IDO used as a transgene.

Finally, there is a well-established but poorly understood correlation

between tryptophan metabolism and autoimmune disorders, as described

20   further in Example 8. Based on the data herein, it is proposed that IDO

suppresses autoreactive T cell activation by degrading tryptophan in local

microenvironments. From this it follows that elevated systemic tryptophan

levels would make it more difficult for macrophages to deplete tryptophan

locally and hence suppress autoreactive T cells; whereas lowered systemic

25   tryptophan levels would favor suppression of autoreactive T cells.

Therefore, methods to lower systemic tryptophan levels by pharmacologic

induction of IDO, administration of tryptophan-degrading enzymes, or *ex

vivo* depletion of plasma tryptophan by tryptophan-degrading enzymes,

could be used in the treatment of autoimmune disorders. Examples of

30   suitable enzymes for lowering tryptophan such as indolyl-3-alkane alpha-

hydroxylase, and means for *ex vivo* treatment of plasma to lower tryptophan

levels which are abnormally elevated are described in U.S. Patent Nos.

5,723,325 and 5,244,807 to Murtfeldt, et al.

Inhibitors of the IDO enzyme (such as 1-methyl-DL-tryptophan, β-

35   (3-benzofuranyl)-DL-alanine, 6-nitro-L-tryptophan and β-[3-

benzo(b)thienyl]-DL-alanine), or the high affinity tryptophan transporter can

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5   be used to simulate T cell mediated immune responses where these would

normally be suppressed by IDO. Applications include using these agents

(systemically or locally administered) to an individual to terminate

pregnancy. Other applications include transfection of the IDO gene (under

an inducible promoter) into tissues and cells prior to or at the time of

10   pregnancy, to reduce the possibility of immune-mediated rejection of the

fetus. Screening of tryptophan levels and/or IDO expression can be used as

indicators of T cell activation or suppression and therefore predictors of

miscarriage or spontaneous abortion.

Based on these studies and the underlying mechanism, it is possible

15   to terminate, or help to sustain in some individuals, pregnancy. To terminate

the pregnancy one administers an effective amount of an inhibitor of IDO, or

transport of tryptophan by the high affinity tryptophan transporter. For

example, an inhibitor such as 1-methyltryptophan at a dosage of 1 g/kg is

effective to cause rejection and killing by macrophages. The dosage may be

20   administered one or more times, as required, to yield the desired effect.

Compounds which Modulate Tryptophan or Tryptophan Metabolite

A number of techniques are known for obtaining compounds which

can be used to modulate tryptophan or tryptophan metabolite levels. The

25   active compounds can be divided into the following categories:

(1)   IDO and other enzymes whos activity can be modulated to

alter tryptophan degradation

(2)   The high affinity sodium-independent extremely selective

tryptophan transporter and other transporters of tryptophan which can

30   increase or decrease typtophan transport into the cell

IDO is well characterized, as discussed above. Bacterial enzymes,

also discussed herein, can also be used to degrade tryptophan. Tryptophan

transporter are described herein and in the literature. Although the following

description is primarily directed towards modulation of IDO activity, the

35   descriptions are equally applicable for modulators of the high affinity

tryptophan transporter, which can be regulated in the same manner as other

17

5   transporter molecules, by turning on or off transport by completely blocking

access to the transporter, or by partially blocking access, for example, by

binding of the transporter with a molecule which competitively binds with

tryptophan, or which has a higher binding affinity or lower dissociation

constant than tryptophan. IDO is an intracellular enzyme. In order for it to

10   affect the extracellular level of tryptophan its substrate must be transported

across the macrophage cell membrane. There are many known amino acid

transport systems in mammalian cells, a number of which accept tryptophan.

However, none of these are specific for tryptophan, so it must compete with

various other amino acids for uptake. To date, no preferential tryptophan

15   transporter has been described in mammalian cells. Data suggests that there

may exist an IFNγ-inducible, high-affinity, tryptophan-selective uptake

system in MCSF-derived macrophages. It is sodium-independent, placing it

in a limited category. The known sodium-independent systems are

summarized in Kakuda *J. Exp. Biol.* 1994;196:93-108; McGivan *Biochem. J.*

20   1994;299:321-324. In some ways the macrophage transport system

resembles system L. However, in cross-competition studies, the tryptophan

uptake system in macrophages shows a 10- to 100-fold higher affinity for

tryptophan than for the next best substrate, phenylalanine, which does not

correspond to any of the known systems. System T, which also accepts

25   tryptophan, has a much lower affinity than the system observed in

macrophages (Zhou J. Biol. Chem. 1990;265:17000-17004). I

         Assays for testing compounds for useful activity can be based solely

on interaction with IDO or enzymes or the transporters involved in

tryptophan metabolism ("the enzymes"), or alternatively, the assays can be

30   based on interaction with the gene sequence encoding the enzymes. For

example, antisense which binds to the regulatory sequences, and/or to the

protein encoding sequences can be synthesized using standard

oligonucleotide synthetic chemistry. The antisense can be stabilized for

pharmaceutical use using standard methodology (encapsulation in a liposome

35   or microsphere; introduction of modified nucleotides that are resistant to

degradation or groups which increase resistance to endonucleases, such as

5   phosphorothiodates and methylation), then screened initially for alteration of

        enzyme activity in transfected or naturally occurring cells which express the

        enzyme, then *in vivo* in laboratory animals. Typically, the antisense would

        inhibit expression. However, sequences which block those sequences which

        "turn off" synthesis can also be targeted

10   Compounds which inhibit IDO are known, as demonstrated by the

        examples, although not previously described for use as immunomodulators.

        Additional compounds which are highly selective can be obtained as follows.

                    Random generation of enzyme or enzyme encoding sequence
                    binding molecules.

15   Molecules with a given function, catalytic or ligand-binding, can be

        selected for from a complex mixture of random molecules in what has been

        referred to as "in vitro genetics" (Szostak, TIBS 19:89, 1992). One

        synthesizes a large pool of molecules bearing random and defined sequences

        and subjects that complex mixture, for example, approximately 10^{15}

20   individual sequences in 100 µg of a 100 nucleotide RNA, to some selection

        and enrichment process. For example, by repeated cycles of affinity

        chromatography and PCR amplification of the molecules bound to the ligand

        on the column, Ellington and Szostak (1990) estimated that 1 in 10^{10} RNA

        molecules folded in such a way as to bind a given ligand. DNA molecules

25   with such ligand-binding behavior have been isolated (Ellington and Szostak,

        1992; Bock et al, 1992).

                    Computer assisted drug design

               Computer modeling technology allows visualization of the three-

        dimensional atomic structure of a selected molecule and the rational design

30   of new compounds that will interact with the molecule. The three-

        dimensional construct typically depends on data from x-ray crystallographic

        analyses or NMR imaging of the selected molecule. The molecular

        dynamics require force field data. The computer graphics systems enable

        prediction of how a new compound will link to the target molecule and allow

35   experimental manipulation of the structures of the compound and target

        molecule to perfect binding specificity. Prediction of what the molecule-

        compound interaction will be when small changes are made in one or both

                                        19

5   requires molecular mechanics software and computationally intensive

computers, usually coupled with user-friendly, menu-driven interfaces

between the molecular design program and the user.

Examples of molecular modelling systems are the CHARMm and

QUANTA programs, Polygen Corporation, Waltham, MA. CHARMm

10   performs the energy minimization and molecular dynamics functions.

QUANTA performs the construction, graphic modelling and analysis of

molecular structure. QUANTA allows interactive construction,

modification, visualization, and analysis of the behavior of molecules with

each other.

15   A number of articles review computer modeling of drugs interactive

with specific proteins, such as Rotivinen, et al., 1988 Acta Pharmaceutica

Fennica 97, 159-166; Ripka, New Scientist 54-57 (June 16, 1988); McKinaly

and Rossmann, 1989 Annu. Rev. Pharmacol. Toxiciol. 29, 111-122; Perry

and Davies, QSAR: Quantitative Structure-Activity Relationships in Drug

20   Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 Proc.

R. Soc. Lond. 236, 125-140 and 141-162; and, with respect to a model

enzyme for nucleic acid components, Askew, et al., 1989 J. Am. Chem. Soc.

111, 1082-1090. Other computer programs that screen and graphically

depict chemicals are available from companies such as BioDesign, Inc.,

25   Pasadena, CA., Allelix, Inc, Mississauga, Ontario, Canada, and Hypercube,

Inc., Cambridge, Ontario. Although these are primarily designed for

application to drugs specific to particular proteins, they can be adapted to

design of drugs specific to regions of DNA or RNA, once that region is

identified.

30   Although described above with reference to design and generation of

compounds which could alter binding, one could also screen libraries of

known compounds, including natural products or synthetic chemicals, and

biologically active materials, including proteins, for compounds which are

inhibitors or activators.

35   *Generation of nucleic acid regulators*

Nucleic acid molecules containing the 5' regulatory sequences of the

20

5   enzyme genes can be used to regulate or inhibit gene expression *in vivo*.

Vectors, including both plasmid and eukaryotic viral vectors, may be used to

express a particular recombinant 5' flanking region-gene construct in cells

depending on the preference and judgment of the skilled practitioner (see,

e.g., Sambrook et al., Chapter 16). Furthermore, a number of viral and

10   nonviral vectors are being developed that enable the introduction of nucleic

acid sequences *in vivo* (see, e.g., Mulligan, 1993 Science, 260, 926-932;

United States Patent No. 4,980,286; United States Patent No. 4,868,116;

incorporated herein by reference). Nucleic acid can be encapsulated in

cationic liposomes which can be injected intravenously into a mammal (Zhu

15   et al., 1993 Science 261, 209-211).

The 5' flanking sequences of the enzyme gene can also be used to

inhibit the expression of the enzyme. For example, an antisense RNA of all

or a portion of the 5' flanking region of the enzyme gene can be used to

inhibit expression of the enzyme *in vivo*. Expression vectors (e.g., retroviral

20   expression vectors) are already available in the art which can be used to

generate an antisense RNA of a selected DNA sequence which is expressed

in a cell (see, e.g., U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286).

Accordingly, DNA containing all or a portion of the sequence of the 5'

flanking region of the enzyme gene can be inserted into an appropriate

25   expression vector so that upon passage into the cell, the transcription of the

inserted DNA yields an antisense RNA that is complementary to the mRNA

transcript of the enzyme gene normally found in the cell. This antisense

RNA transcript of the inserted DNA can then base-pair with the normal

mRNA transcript found in the cell and thereby prevent the mRNA from

30   being translated. It is of course necessary to select sequences of the 5'

flanking region that are downstream from the transcriptional start sites for

the enzyme gene to ensure that the antisense RNA contains complementary

sequences present on the mRNA.

Antisense RNA can be generated *in vitro* also, and then inserted into

35   cells. Oligonucleotides can be synthesized on an automated synthesizer

(e.g., Model 8700 automated synthesizer of Milligen-Biosearch, Burlington,

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5   MA or ABI Model 380B). In addition, antisense deoxyoligonucleotides have
       been shown to be effective in inhibiting gene transcription and viral
       replication (see e.g., Zamecnik et al., 1978 Proc. Natl. Acad. Sci. USA 75,
       280-284; Zamecnik et al., 1986 Proc. Natl. Acad. Sci., 83, 4143-4146;
       Wickstrom et al., 1988 Proc. Natl. Acad. Sci. USA 85, 1028-1032; Crooke,

10   1993 FASEB J. 7, 533-539. Inhibition of expression of a gene by antisense
       oligonucleotides is possible if the antisense oligonucleotides contain
       modified nucleotides (see, e.g., Offensperger et. al., 1993 EMBO J. 12,
       1257-1262 (in vivo inhibition of duck hepatitis B viral replication and gene
       expression by antisense phosphorothioate oligodeoxynucleotides);

15   Rosenberg et al., PCT WO 93/01286 (synthesis of sulfurthioate
       oligonucleotides); Agrawal et al., 1988 Proc. Natl. Acad. Sci. USA 85, 7079-
       7083 (synthesis of antisense oligonucleoside phosphoramidates and
       phosphorothioates to inhibit replication of human immunodeficiency virus-
       1); Sarin et al., 1989 Proc. Natl. Acad. Sci. USA 85, 7448-7794 (synthesis of

20   antisense methylphosphonate oligonucleotides); Shaw et al., 1991 Nucleic
       Acids Res 19, 747-750 (synthesis of 3' exonuclease-resistant
       oligonucleotides containing 3' terminal phosphoroamidate modifications);
       incorporated herein by reference).

            The sequences of the 5' flanking region of enzyme gene can also be
25   used in triple helix (triplex) gene therapy. Oligonucleotides complementary
       to gene promoter sequences on one of the strands of the DNA have been
       shown to bind promoter and regulatory sequences to form local triple nucleic
       acid helices which block transcription of the gene (see, e.g., 1989 Maher et
       al., Science 245, 725-730; Orson et al., 1991 Nucl. Acids Res. 19, 3435-

30   3441; Postal et al., 1991 Proc. Natl. Acad. Sci. USA 88, 8227-8231; Cooney
       et al., 1988 Science 241, 456-459; Young et al., 1991 Proc. Natl. Acad. Sci.
       USA 88, 10023-10026; Duval-Valentin et al., 1992 Proc. Natl. Acad. Sci.
       USA 89, 504-508; 1992 Blume et al., Nucl. Acids Res. 20, 1777-1784; 1992
       Grigoriev et al., J. Biol. Chem. 267, 3389-3395.

35   Both theoretical calculations and empirical findings have been
       reported which provide guidance for the design of oligonucleotides for use in

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5   oligonucleotide-directed triple helix formation to inhibit gene expression.

For example, oligonucleotides should generally be greater than 14

nucleotides in length to ensure target sequence specificity (see, e.g., Maher et

al., (1989); Grigoriev et al., (1992)). Also, many cells avidly take up

oligonucleotides that are less than 50 nucleotides in length (see e.g., Orson et

10   al., (1991); Holt et al., 1988 Mol. Cell. Biol. 8, 963-973; Wickstrom et al.,

1988 Proc. Natl. Acad. Sci. USA 85, 1028-1032). To reduce susceptibility to

intracellular degradation, for example by 3' exonucleases, a free amine can

be introduced to a 3' terminal hydroxyl group of oligonucleotides without

loss of sequence binding specificity (Orson et al., 1991). Furthermore, more

15   stable triplexes are formed if any cytosines that may be present in the

oligonucleotide are methylated, and also if an intercalating agent, such as an

acridine derivative, is covalently attached to a 5' terminal phosphate (e.g., via

a pentamethylene bridge); again without loss of sequence specificity (Maher

et al., (1989); Grigoriev et al., (1992)).

20   Methods to produce or synthesize oligonucleotides are well known in

the art. Such methods can range from standard enzymatic digestion followed

by nucleotide fragment isolation (see e.g., Sambrook et al., Chapters 5, 6) to

purely synthetic methods, for example, by the cyanoethyl phosphoramidite

method using a Milligen or Beckman System 1Plus DNA synthesizer (see

25   also, Ikuta et al., in Ann. Rev. Biochem. 1984 53, 323-356 (phosphotriester

and phosphite-triester methods); Narang et al., in Methods Enzymol., 65,

610-620 (1980) (phosphotriester method).

Preparation of Enzyme Fragments

Compounds which are effective for blocking binding of the enzyme

30   can also consist of fragments of the enzymes, expressed recombinantly and

cleaved by enzymatic digest or expressed from a sequence encoding a

peptide of less than the full length enzyme. It is a routine matter to make

appropriate enzyme fragments, and test for inhibition of activity of the

enzyme in the presence of the fragments. The preferred fragments are of

35   human origin, in order to minimize potential immunological response. The

peptides can be as short as five to eight amino acids in length and are easily

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5   prepared by standard techniques. They can also be modified to increase *in vivo* half-life, by chemical modification of the amino acids or by attachment to a carrier molecule or inert substrate. The peptides can also be conjugated to a carrier protein such as keyhole limpet hemocyanin by its N-terminal cysteine by standard procedures such as the commercial Imject kit from

10   Pierce Chemicals or expressed as a fusion protein, which may have increased efficacy. As noted above, the peptides can be prepared by proteolytic cleavage of the enzymes, or, preferably, by synthetic means. These methods are known to those skilled in the art. An example is the solid phase synthesis described by J. Merrifield, 1964 J. Am. Chem. Soc. 85, 2149, used in U.S.

15   Patent No. 4,792,525, and described in U.S. Patent No. 4,244,946, wherein a protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of a peptide starting from the C-terminus of the peptide. Other methods of synthesis are described in U.S. Patent No. 4,305,872 and 4,316,891. These methods can be used to synthesize peptides having identical sequence to the

20   enzymes described herein, or substitutions or additions of amino acids, which can be screened for activity as described above.

       The peptide can also be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric

25   acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases

30   such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

       Peptides containing cyclopropyl amino acids, or amino acids derivatized in a similar fashion, can also be used. These peptides retain their original activity but have increased half-lives *in vivo*. Methods known for modifying amino acids, and their use, are known to those skilled in the art,

35   for example, as described in U.S. Patent No. 4,629,784 to Stammer.

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5   *Pharmaceutical Compositions*

Compounds which alter enzyme activity and/or tryptophan transport
are preferably administered in a pharmaceutically acceptable vehicle.
Suitable pharmaceutical vehicles are known to those skilled in the art. For
parenteral administration, the compound will usually be dissolved or
10   suspended in sterile water or saline. For enteral administration, the
compound will typically be administered in a tablet or capsule, which may be
enteric coated, or in a formulation for controlled or sustained release. Many
suitable formulations are known, including polymeric or protein
microparticles encapsulating drug to be released, ointments, gels, or
15   solutions which can be used topically or locally to administer drug, and even
patches, which provide controlled release over a prolonged period of time.
These can also take the form of implants. For example prevention of
pregnancy could be obtained by administration intravaginally of a compound
increasing tryptophan, for example, by inhibiting IDO, thereby resulting in
20   killing of the sperm or fertilized egg almost immediately.

Generation of Transgenic Animals for Screening

With the knowledge of the cDNA encoding the enzyme and
regulatory sequences regulating expression thereof, it is possible to generate
transgenic animals, especially rodents, for testing the compounds which can
25   alter enzyme expression, translation or function in a desired manner.

There are basically two types of animals which are useful: those not
expressing functional enzyme, and those which overexpress enzyme, either
in those tissues which already express the protein or in those tissues where
only low levels are naturally expressed. The animals in the first group are
30   preferably made using techniques that result in "knocking out" of the gene
for enzyme, although in the preferred case this will be incomplete, either
only in certain tissues, or only to a reduced amount. These animals are
preferably made using a construct that includes complementary nucleotide
sequence to the enzyme gene, but does not encode functional enzyme, and is
35   most preferably used with embryonic stem cells to create chimeras. Animals
which are heterozygous for the defective gene can also be obtained by

25

5   breeding a homozygote normal with an animal which is defective in
       production of enzyme.

               The animals in the second group are preferably made using a
       construct that includes an unregulated promoter or one which is modified to
       increase expression as compared with the native promoter. The regulatory

10   sequences for the enzyme gene can be obtained using standard techniques
       based on screening of an appropriate library with the cDNA encoding
       enzyme. These animals are most preferably made using standard
       microinjection techniques.

               These manipulations are performed by insertion of cDNA or genomic

15   DNA into the embryo using microinjection or other techniques known to
       those skilled in the art such as electroporation, as described below. The
       DNA is selected on the basis of the purpose for which it is intended: to
       inactivate the gene encoding an enzyme or to overexpress or express the
       gene encoding enzyme. The enzyme encoding gene can be modified by

20   homologous recombination with a DNA for a defective enzyme, such as one
       containing within the coding sequence an antibiotic marker, which can then
       be used for selection purposes.

               Animals suitable for transgenic experiments can be obtained from
       standard commercial sources. These include animals such as mice and rats

25   for testing of genetic manipulation procedures, as well as larger animals such
       as pigs, cows, sheep, goats, and other animals that have been genetically
       engineered using techniques known to those skilled in the art. These
       techniques are briefly summarized below based principally on manipulation
       of mice and rats. The procedures for manipulation of the embryo and for

30   microinjection of DNA are described in detail in Hogan et al. Manipulating
       the mouse embryo, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
       (1986), the teachings of which are incorporated herein. These techniques are
       readily applicable to embryos of other animal species, and, although the
       success rate is lower, it is considered to be a routine practice to those skilled

35   in this art. Methods for the culturing of ES cells and the subsequent
       production of transgenic animals, the introduction of DNA into ES cells by a

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5   variety of methods such as electroporation, calcium phosphate/DNA

       precipitation, and direct injection are described in detail in <u>Teratocarcinomas</u>

       <u>and embryonic stem cells, a practical approach</u>, ed. E.J. Robertson, (IRL

       Press 1987), the teachings of which are incorporated herein. Selection of the

       desired clone of transgene-containing ES cells is accomplished through one

10   of several means. In cases involving sequence specific gene integration, a

       nucleic acid sequence for recombination with the enzyme gene or sequences

       for controlling expression thereof is co-precipitated with a gene encoding a

       marker such as neomycin resistance. Transfection is carried out by one of

       several methods described in detail in Lovell-Badge, in <u>Teratocarcinomas</u>

15   <u>and embryonic stem cells, a practical approach</u>, ed. E.J. Robertson, (IRL

       Press 1987) or in Potter et al <u>Proc. Natl. Acad. Sci. USA</u> 81, 7161 (1984).

       Calcium phosphate/DNA precipitation, direct injection, and electroporation

       are the preferred methods. DNA molecules introduced into ES cells can also

       be integrated into the chromosome through the process of homologous

20   recombination, described by Capecchi, (1989).

             Once the transgenic animals are identified, lines are established by

       conventional breeding and used as the donors for tissue removal and

       implantation using standard techniques for implantation into humans.

                      Therapeutic Applications

25   The examples described herein demonstrate that tryptophan depletion

       and/or catabolism is the mechanism by which macrophages suppress T cell

       activation, using *in vitro* human and *in vivo* transgenic mouse models;

       defines the developmental regulation of the IDO gene during hematopoietic

       differentiation of macrophages and dendritic cells; and defines the functional

30   characteristics and developmental regulation of the tryptophan transport

       pathway in immunosuppressive macrophages. These studies define a new

       and previously unsuspected role for tryptophan metabolism to immune

       regulation.

             Pharmaceutical compositions based on these discoveries rely on

35   either increasing tryptophan concentrations (and thereby decreasing

       tryptophan metabolite concentrations) or decreasing tryptophan

5   concentrations (and thereby increasing tryptophan metabolite

concentrations). Inhibitors of IDO increase tryptophan concentrations (and

thereby decrease tryptophan metabolite concentrations), increasing T cell

activation. IDO decreases tryptophan concentrations (and thereby increases

tryptophan metabolite concentrations), decreasing T cell activation.

10   Inhibitors of the IDO enzyme, such as 1-methyl-DL-tryptophan, β-(3-

benzofuranyl)-DL-alanine, β-[3-benzo(b)thienyl]-DL-alanine, and 6-nitro-L-

tryptophan, can be used to simulate T cell mediated immune responses where

these would normally be suppressed by IDO. Inhibitors of other enzymes

involved in tryptophan metabolism or degradation can also be used.

15   Applications include using these agents (systemically administered) as

vaccine adjuvants to increase the immunogenicity of administered antigens;

using the agents for immunotherapy of malignancy, including as an adjuvant

for therapeutic tumor-cell vaccines, and as an adjunct to cytokine-based

immunotherapy of malignancy; and using these agents to help reverse the

20   immunosuppressed state found in AIDS since IDO is known to be induced

during HIV infection; co-administering IDO inhibitors with HIV vaccine to

enhance the efficacy of HIV vaccines since IDO is induced in macrophage-

lineage cells by HIV infection; and generally for any setting in which IDO

mediates unwanted suppression of a desired immune response.

25   Alternatively, recombinant IDO can be used as a means of systemic

or local T cell suppression. Applications include transfection of the IDO

gene (under an inducible promoter) into tissues and cells prior to

allotransplantation, in order to allow them to protect themselves from

rejection by the host, for example, by retroviral or other transfer of the IDO

30   gene into allogeneic solid-organ grafts (kidney, etc.); by transgenic

expression of the gene in porcine or other animals designed as donors for

xeno-transplantation, to prevent rejection; or by retroviral or other transfer

into cell preparations intended for transplantation (eg, pancreatic islet cells

for therapy of diabetes mellitus).

35   Applications also include using these agents (systemically or locally

administered) to an individual to terminate pregnancy. Other applications

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5   include transfection of the IDO gene (under an inducible promoter) into

tissues and cells prior to or at the time of pregnancy, to reduce the possibility

of immune-mediated rejection of the fetus. To terminate the pregnancy one

administers an effective amount of an inhibitor of IDO, or transport of

tryptophan by the high affinity tryptophan transporter. For example, an

10   inhibitor such as 1-methyltryptophan at a dosage of 1 g/kg is effective to

cause rejection and killing by macrophages. The dosage may be

administered one or more times, as required, to yield the desired effect.

Screening of tryptophan levels and/or IDO expression can be used as

indicators of T cell activation or suppression. Screening of tryptophan levels

15   and/or IDO expression can be used as indicators of T cell activation or

suppression and therefore predictors of miscarriage or spontaneous abortion.

Testing for IDO expression tumor biopsies can be used to determine the

suitability of the cancer for IDO-targeted therapy.

The present invention will be further understood by reference to the

20   following non-limiting examples.

Example 1:   Macrophage-induced suppression.

As described in Munn, et al. J. Immunol. 1996;156:523-532, a

number of possible mechanisms for macrophage-induced suppression were

initially examined. None of those tested, including the fas/fas-L system,

25   nitric oxide, free radicals, prostaglandins, and inhibitory cytokines, could

account for the phenomenon. Studies demonstrated the following regarding

macrophage inhibition of T cell proliferation:

(1)   MCSF-derived macrophages inhibit the mitogen-induced

proliferation of T cells, and this is a dominant effect -- i.e., it is not

30   rescued by adding non-inhibitory APCs.

(2)   The T cells initiate early activation, as assessed by activation-related

G1 phase genes (IL-2 receptor, cdc2, cyclin A), but arrest prior to the

first G1/S boundary. This arrest is not a stable condition, and most of

the lymphocytes progressively die off.

35   (3)   The suppressive effect was developmentally regulated by MCSF and

IFNγ. It was not present in fresh monocytes, emerged progressively

29

5   over 4-5 days of differentiation in MCSF, and was prevented if the monocytes were exposed to IFNγ (even transiently) prior to differentiation. IFNγ has a dual action in macrophage differentiation. If monocytes are exposed to IFNγ *prior* to differentiation it functions as a developmental regulator, and the monocytes will not

10   subsequently acquire the suppressive phenotype - even in the presence of MCSF. However, if macrophages are exposed to IFNγ *after* differentiation, it causes activation but does not alter the MCSF-induced phenotype. Thus, the nature of the response to IFNγ is determined by the developmental state of the macrophage.

15 (4)   There was no evidence for participation of nitric oxide, reactive oxygen species, TNF, TGFβ, prostaglandins, IL-1R antagonist, or IL-10. The mechanism of inhibition appeared contact dependent. Inhibition could not be reproduced by conditional medium (up to 50% v/v), and was abrogated when T cells were separated from

20   macrophages by a semipermeable membrane. The contact-dependence is understandable in light of the data indicating a requirement for CD40/CD40L in order to produce full activation of tryptophan degradation.

Example 2:   Tryptophan degradation by MCSF-derived Macrophages

25   *Medium in macrophage-lymphocyte co-cultures is selectively depleted of tryptophan.* Conditioned medium was generated from co-cultures of macrophages plus T cells activated with mitogen (two different mitogens, anti-CD3 antibody and SEB, gave equivalent results; the data presented are from anti-CD3). Fresh lymphocytes were suspended in

30   conditioned medium and activated with additional mitogen. Care was taken to exclude all traces of fresh medium.

       As shown in Figure 1, conditioned medium completely failed to support T cell proliferation (less than 1% of the proliferation in fresh medium). In contrast, control conditioned media from macrophages alone,

35   from co-cultures of macrophages and T cells without mitogen, and from T cells activated with fresh monocytes instead of macrophages, all supported T

30

5   cell proliferation comparably to fresh medium (90-140%) of control, n=3-4

       in each group, p=NS by ANOVA, see Munn J. Immunol. 1996;156:523-532

       for representative data.

               Selective add-back studies showed that the deficiency lay in an

       essential amino acid. Supplementation with individual amino acids (Figure

10   1) showed that tryptophan was depleted in conditioned medium. The

       addition of tryptophan to conditioned medium fully restored T cell

       proliferation, indicating that tryptophan was the only deficient component.

       Consistent with this, HPLC analysis of conditioned media showed that the

       levels of other essential amino acids were similar to fresh medium (some

15   concentrations were higher, presumably due to protein catabolism).

               As shown in Figure 2, titration of reagent tryptophan in conditioned

       medium gave a half-maximal concentration of approximately 500 nM for T

       cell proliferation (compared to a normal level in RPMI of 25 μM). These

       studies also showed that as little as 50 nM was sufficient to initiate low but

20   readily detectable T cell proliferation, indicating that tryptophan had been

       reduced below this range. Consistent with this, direct measurement of

       tryptophan in conditioned medium was below the detection limit for our

       assay, which was 50 nM.

               Depletion of tryptophan is rapid. Macrophages were co-cultured

25   with T cells and mitogen for 24 hrs to allow up-regulation of the tryptophan-

       depletion pathway, then fresh medium was added and the kinetics of

       elimination measured. As shown in Figure 3, tryptophan was eliminated by

       first-order kinetics with a half-life of approximately 2 hours. The initial rate

       of elimination (when tryptophan was not limiting) was up to 20,000

30   pmol/10^6 cells/hr. By way of comparison, fresh monocytes stimulated with

       IFNγ (a standard model of IDO induction) degraded tryptophan at

       approximately 200 pmol/10^6 cells/hr, which is consistent with the literature.

       This rapid rate of tryptophan depletion could not be attributed to

       consumption by cellular metabolism. Despite their high metabolic activity,

35   macrophages alone depleted tryptophan at a barely detectable rate (less than

       5% of the activated rate, Figure 3). It also did not reflect sequestration of

5   free tryptophan within macrophages, since the preliminary validation studies
     had shown that tryptophan was undetectable in culture supernatants whether
     macrophages were intact or lysed prior to performing the assay.

Example 3:   Inhibition of Tryptophan degradation by MCSF-derived
Macrophages prevents Suppression of T cells.

10   *Inhibition of tryptophan degradation prevents Macrophage-mediated*
     suppression of T cells. The data predicts that pharmacologic inhibitors of
     IDO should prevent suppression. One potent inhibitor of IDO is the 1-
     methyl derivative of tryptophan (Cady, et al. Arch. Biochem. Biophys.
     1991;291:326-333). As shown in Figure 4A, the presence of 1-methyl-
15   tryptophan effectively prevented macrophages from suppressing T cell
     activation. These data were selected in order to clearly demonstrate that
     suppression can be abrogated, but in most experiments the effect of 1-
     methyl-tryptophan has been less complete (typically 59-60% reversal of
     suppression). This was not unexpected, given the extremely high level of
20   enzyme activity and the fact that 1-methyl-tryptophan is a competitive, rather
     than an irreversible, inhibitor. In order to fully abrogate macrophage-
     mediated suppression it will be necessary to simultaneously block the
     induction of the enzyme and to pharmacologicially inhibit its activity.

             Macrophages can overcome high concentrations of tryptophan. A
25   second test of the mechanism was to supplement the culture medium with
     additional tryptophan to reverse the inhibitory effect of IDO. Tryptophan
     supplementation alone was never as effective as 1-methyl-tryptophan, and
     sometimes was entirely ineffective, and as shown in Figure 4B, appears to be
     related to the seeding density of macrophages and T cells. At low densities,
30   supplemental tryptophan effectively reversed suppression, but at high
     densities the macrophages remained able to suppress. The effect of seeding
     density is believed to reflect the limited ability of diffusion to deliver
     tryptophan to the T cells and macrophages.

Example 4:   Effect of Cytokines on Macrophage Tryptophan
35   **Degradation.**

             Macrophage tryptophan degradation is synergistically induced by

32

5   *signals of early T cell activation.* The rapid depletion of tryptophan by
     macrophages was not induced by resting T cells, but occurred only when the
     T cells attempted to activate (cf. Figure 3). This implied that the
     macrophages were detecting some sign of T cell activation which triggered
     tryptophan degradation. In light of the studies implicating IFNγ as a major

10   regulator of the IDO gene, it was predicted that T cell-derived IFNγ would be
     the signal for tryptophan degradation. However, when dose-response
     titration was performed of recombinant IFNγ on MCSF-derived
     macrophages, it was found that IFNγ concentrations of 100 U/ml or more
     were required for maximal induction, and even then the level of enzyme

15   activity did not reach those achieved by co-culture with activated T cells.

     CD40L is upregulated early in T cell activation and it is known to act
     synergistically with IFNγ in signaling macrophages for other functions. The
     response of MCSF-derived macrophages to IFNγ in the presence or absence
     of recombinant CD40L (prepared as a homo-trimer in order to signal via

20   CD40 in soluble form, gift of Bill Fanslow, Immunex Corporation) was
     compared. The addition of CD40L alone had little effect on enzyme activity,
     but as shown in Figure 5 it displayed marked synergy when combined with
     IFNγ. The effect of CD40L was to shift the dose-response curve of IFNγ one
     to two orders of magnitude, so that significant response now occurred at

25   IFNγ concentrations as low as 1 U/ml. Thus, two signals normally delivered
     by T cells to APC early in the course of activation combined to induce
     tryptophan degradation in MCSF-derived Mφs.

     *The responsiveness of tryptophan metabolism to IFNγ and CD40L is
     developmentally regulated.* It was then determined if all monocyte-derived

30   cells degraded tryptophan in response to these signals, or whether it was
     specifically associated with the MCSF phenotype. To generate a non-
     suppressive phenotype for comparison, monocytes were cultured for 5 days
     GMCSF+IL-4, which produces cells that closely resemble tissue dendritic
     cells, and which are potent activators of T cells. For clarity, these cells are

35   referred to as dendritic cells and MCSF-derived cells as macrophages. As
     shown in Figure 6, dendritic cells showed minimal tryptophan degradation in

                                          33

5 response to IFNγ and CD40L, in contrast to MCSF-derived macrophages.

Thus, the response to IFNγ was not simply a feature of differentiated

monocytes, but was regulated by the cytokine milieu present during terminal

differentiation. Interestingly, when the cytokines were added sequentially,

the effect of CD40L on dendritic cells was to further inhibit any residual

10 induction of tryptophan degradation in response to IFNγ, exactly the opposite

of its effect on macrophages.

Example 5:   The tryptophan transport system in Macrophages

Transport of tryptophan into Macrophages is developmentally

regulated. Whether the upregulation of tryptophan degradation was

15 accompanied by increased or decreased tryptophan transport into

macrophages was then determined. As shown in Figure 7A, following

differentiation MCSF-derived macrophages showed a markedly increased

ability to take up tryptophan, 10 to 20-fold greater than fresh monocytes,

which was further induced by activation with IFNγ.

20 *Tryptophan transport in MCSF-derived Macrophages shows a*

preferential affinity for tryptophan. To characterize the tryptophan transport

system, whether uptake was dependent on the sodium gradient was then

determined. Preliminary studies had shown that tryptophan transport in fresh

monocytes was sodium dependent (less than 10% of transport was sodium-

25 independent). However, as shown in Figure 7B, the majority of the

tryptophan transport in macrophages was independent of sodium. This

sodium-independent system was then characterized using cross-competition

studies. As expected, T cells showed a pattern consistent with system L,

with tryptophan uptake being inhibited by aromatic and neutral amino acids,

30 but not lysine or arginine (Figure 8A). In contrast, however, tryptophan

uptake by MCSF macrophages (Figure 8B) was only partially inhibited by

neutral amino acids, and even phenylalanine could not fully block it.

As shown in Figure 9, titration studies indicated that phenylalanine

(the best competitor) was 10- to 100-fold less effective than tryptophan in

35 competing for uptake, based on relative IC_{50}. Tyrosine, leucine, and the

system L-specific substrate 2-amino-2-norborane-carboxylic acid (BCH)

5   were even less effective competitors. Significantly, when system L was

inhibited by 8 mM BCH, there still remained 30-50% of the total tryptophan

transport available (depending on the experiment). Taken together these data

suggested that a high-affinity, non-system L transport pathway might be

present.

10   **Example 6:   In vivo studies of the role of IDO and tryptophan**

degradation on activation of T cells.

Peripheral deletion of autoreactive T cells in vivo is accompanied by

evidence of tryptophan degradation. Whether there was evidence that IDO

was involved in peripheral tolerance *in vivo* was then assessed. Since IDO

15   activity at the single cell level cannot be measured *in vivo*, a model in which

a large number of autoreactive T cells underwent synchronous activation and

deletion was needed. This would allow one to follow the kynurinine

breakdown products released systemically when IDO degrades tryptophan

(Meyer, et al. *J. Lab. Clin. Med.* 1995;126:530-540). Dr. Mellor had

20   developed such a model by creating mice transgenic for a T cell receptor

recognizing the H-2K^b (MHC class I) antigen. When these T cells are

adoptively transferred into a syngeneic animal made transgenic for the target

antigen, they become functionally autoreactive. As described in Tarazona, et

al. *Intl. Immunol.* 1996;8:351-358, these autoreactive T cells undergo a

25   transient period of activation, but then are rapidly eliminated (day 4-5)

without causing disease. This occurs despite the fact that they are not subject

to rejection by the host (being syngeneic), and are eliminated only in animals

where they encounter their antigen. This therefore represents a useful model

of synchronous deletion of autoreactive T cells.

30   Splenocytes from T cell receptor-transgenic mice (4 x 10^7 cells) were

adoptively transferred by tail vein injection into antigen-transgenic mice. On

day 3 (at the beginning of the rapid expansion phase), the recipient animals

were sacrificed and serum was obtained. Pre-transfer sera were used as

controls. As shown in Figure 10, mice which had received autoreactive T

35   cells showed a marked elevation in serum kynurinine compared to controls.

Thus, the expansion and elimination of autoreactive T cells *in vivo* was

5   accompanied by evidence of significant tryptophan catabolism, consistent
        with activation of IDO.

                Whether *in vivo* inhibition of the IDO enzyme with 1-methyl
        tryptophan (as described *in vitro* in Figure 4A) would alter the kinetics of
        activation or elimination of autoreactive T cells in the mouse model was then

10   measured. As shown in Figure 11, continuous infusion of 1-methyl
        tryptophan (via subcutaneously implanted timed-release pellets, 1000
        mg/kg/day) resulted in significantly enhanced activation of the adoptively
        transferred T cells. These results strongly support a role for IDO in
        suppressing T cell activation *in vivo*.

15   **Example 7:   MCSF and tissue Macrophages.**

                Clinically, there exists an ill-defined association between altered
        tryptophan metabolism and autoimmune disorders. Patients receiving L-5-
        hydroxytryptophan for neurologic disorders experienced a high frequency of
        a scleroderma-like illness (Sternberg *N. Engl. J. Med.* 1980;303:782-787).

20   Individuals who ingested certain preparations of L-tryptophan contaminated
        with toxic tryptophan derivatives (1-1' ethylidenebis-[tryptophan])
        experienced the eosinphilia-myalgia syndrome (Belonga *N.E. Engl. J. Med.*
        1990;323:357-365; Mayeno *Science* 1990;250:1707-1708), which included
        excessive T cell activation. There is even a case report of simple dietary

25   supplementation with tryptophan leading to multiple autoimmune disorders
        (Morgan, et al. *Br. J. Dermatol.* 1993;128:581-583).

                The osteopetrotic (*op/op*) mouse lacks functional MCSF, and has a
        selective deficiency in specific sublets of tissue macrophages (Ceccini, et al.
        Development 1994;120:1357-1372). It has normal dendritic cell

30   development and normal T cell responses to foreign antigens (Begg J. Exp.
        Med. 1993;177:237-242) indicating that these functions are not MCSF-
        dependent. Interestingly, in one well-established model of spontaneous
        autoimmunity, the NOD mouse, the macrophages have been shown to have a
        defective proliferative response to MCSF, and fail to undergo MCSF-

35   induced terminal differentiation (Serreze, et al. J. Immunol. 1993;150:2534-
        2543). This led to speculate that autoimmune NOD mice lack a specific,

5   MCSF-induced tolerogenic macrophage subset. The *in vitro* studies show

that MCSF is a growth factor for both immunosuppressive and inflammatory

macrophages, and that when inflammatory cytokines are present they act

dominantly over MCSF. Thus, in the presence of dysregulated inflammatory

cytokines, the addition of MCSF would be expected to exacerbate rather than

10   reduce the disorder (Moore, et al. J. Immunol. 1996;157:433-440).

         Based on the preliminary data, it was proposed that IFNγ induces

IDO in certain macrophage and thereby triggers T cell suppression. This is a

somewhat surprising role for IFNγ, which is generally considered pro-

inflammatory rather than immunoregulatory. However, mice with targeted

15   disruptions of the gene for either IFNγ (Krakowsk, et al. Eur. J. Immunol.

1996;26:1641-1646) or the IFNγ receptor (Willenborg, et al. J. Immunol.

1996;157:3223-3227) demonstrate an unexpected hyper-susceptibility to

experimental autoimmune encephalomyelitis (EAE). Mice normally

resistant to this induced autoimmune disorder are rendered susceptible in the

20   absence of IFNγ, and the disease is fatal and uncontrolled. Adoptive transfer

studies showed that the requirement for IFNγ lay with an IFNγ-responsive

regulatory cell, not with the autoreactive T cells. In contrast, these mice fail

to mount an appropriate inflammatory response to intracellular pathogens

(Dalton, et al. Science 1993;259:1739-1742; Kamijo, et al. J. Exp. Med

25   1993;178:1435-1440). Thus, IFNγ is pro-inflammatory in infectious disease,

but is immunoregulatory in at least one model of autoimmunity. This is

consistent with the hypothesis that some macrophages respond to IFNγ as a

signal to inhibit T cell activation.

         The following examples demonstrate that 1-methyl-L-tryptophan, an

30   inhibitor of L-tryptophan degradation, administered during murine

pregnancy induces loss of allogeneic (mother and fetus are genetically

different) conceptus 2-3 days after blastocyst implantation in uterus.

Syngeneic conceptus are not affected by this treatment.

Example 8:   The mIDO gene is transcribed in epididymis and in

35   **conceptus during pregnancy.**

         RT-PCR analyses on RNA extracted from a panel of mouse tissues

5   was conducted to examine the extent of mIDO gene transcription in mice.

mIDO transcripts were detected in RNA samples from mouse epididymis but

not from muscle, heart, bone-marrow, spleen, peripheral lymph nodes,

kidney, liver, brain, intestine or lung. PCR products were generated by RT-

PCR amplification from RNA samples prepared from pooled syngeneic (s)

10   or allogeneic (a) conceptus. RT-PCR amplification of murine α-actin

transcripts was conducted on each RNA sample to verify RNA integrity.

Methods: Female CBA mice were mated with syngeneic or allogeneic (B6)

male mice. Females were inspected daily (am) for vaginal plugs; the

morning plugs were detected was taken as 0.5 dpc (days *post coitus*). All

15   conceptus dissected from each pregnant female were pooled, snap frozen on

liquid nitrogen and used or prepare total RNA using standard procedures by

homogenization in RNA-STAT 60 solution (Tel-TestB Inc.). Transcripts of

the murine IDO gene (Habara-Ohkubo, et al., 1991 Gene 105) were detected

by RT-PCR using forward (GTACATCACCATGGCGTATG) and reverse

20   (GCTTTCGTCAAGTCTTCATTG) oligonucleotide primers which

generated PCR products of the expected size (740bp. RT-PCR conditions

used were 48°C, 45 min./94°C, 2 min. (1 cycle); 94°C, 30 sec./58°C, 1

min./68°C, 2 min. (40 cycles); 68°C 5 min. (1 cycle). PCR products were

fractionated on a 1.5% agarose/TBE gel containing ethidium bromide and

25   were visualized by UV fluorescence and images of gels were recorded as a

digital bitmaps using a high definition digital fluorescence and images of

gels were recorded as a digital bitmaps using a high definition digital camera.

RT-PCR amplification of the murine α-actin gene (480bp) was performed in

parallel to verify RNA integrity.

30   In all cases, PCR products from the α-actin gene were detected

indicating that RNA samples were not degraded.

          IDO gene expression has been described in human placental

trophoblast cells. To examine whether IDO transcription occurs during

murine pregnancy, RNA samples were prepared from uterus and conceptus

35   of pregnant mice at various stages of gestation. Female mice (CBA) were

mated to syngeneic (CBA) or allogeneic C57BL/6 (B6) male mice and

5   inspected daily for vaginal plugs. The morning the plug was detected was

taken as 0.5 days post coitus (dpc). RNA samples were prepared from

dissected components of conceptus; embryos, decidua (extra-embryonic

trophoblast plus maternal uterus) and from uterus tissues. mIDO gene

transcripts were detected in conceptus, but not uterus from syngeneic and

10   allogeneic conceptus at early (7.5dpc, 9.5dpc) gestation times. mIDO

transcripts were not detected in embryo RNA at 9.5dpc suggesting that

decidual tissues were the site of IDO expression in the conceptus. At later

gestation times (10.5 and 13.5dpc) mIDO transcripts were detected in

allogeneic conceptus but not in syngeneic conceptus. RNA was prepared

15   from decidual tissues dissected separately from embryos. mIDO transcripts

were not detected in RNA from embryos at 10.5 or 13.5dpc. From these data

it was concluded that mIDO transcription occurs in decidual tissues of all

conceptus from early gestation times (7.5-9.5dpc).

Example 9:   Loss of allogeneic conceptus occurs in the presence of an

20   **inhibitor of IDO enzyme activity.**

A study was conducted to test whether IDO enzyme activity

contributes to survival of fetal allografts by treating pregnant female mice

with 1-methyl-L-tryptophan, an inhibitor of IDO enzyme function. Female

(CBA) mice were mated with syngeneic (CBA) or allogeneic (B6) male

25   mice. On 4.5 days post coitus ("dpc") two slow-release pellets impregnated

with 1-methyl-L-tryptophan (1g/kg/day) were surgically implanted under the

dorsal skin of pregnant mice. Control mice were treated with pellets not

impregnated with 1-methyl-L-tryptophan (placebo groups). After surgery,

mice were sacrificed and their uterus examined macroscopically and

30   microscopically at various stages of gestation.

Based on comparison with extensive breeding records from the MCG

Transgenic Unit fecundity rates for mouse colonies bred at MCG are 6.8

(CBA x CBA) and 6.4 (CBA x B6) pups per female at parturition. The same

procedure was used to assess embryo survival and development in the mating

35   combinations presented in Table 2. A large cohort of untreated female CBA

mice mated with GK transgenic male mice were examined and are included

5   in Table 1 to show that the mean number of conceptus (at parturition) is

identical to the [CBA x B6] mating combination.

Results are summarized in Table 1.

Table 1 -	Inhibition of IDO activity induces rejection of allogeneic conceptus				
Mating Genotype conceptus	Gestation Stage	Mean No. conceptus/♀; (No. ♀ treated)		Appearance of	
♀ x ♂	(dpc)	Inhibitor	Placebo	Inhibitor	Placebo
CBA x CBA	6.5-8.5	7.3(3)	7.5(2)	normal	normal
	15.5	6.7(6)	6.5(6)	normal	normal
CBA x B6	6.5	7.3(3)	8.5(2)	normal	normal
	7.5	4.5* (6)	8.0 (4)	majority inflamed	normal
	8.5/9.5	0.5* (17)	7.3 (6)	all inflamed	normal
	11.5-15.5	0* (7)	5.8 (4)	-	normal

*-p<0.05 by ANOVA compared to placebo controls at each time point

10   Treatment with 1-methyl-L-tryptophan had a profound affect on

development of allogeneic conceptus. Syngeneic and allogeneic conceptus

in mice treated with 1-methyl-L-tryptophan were found in normal numbers,

were all healthy and were at the appropriate stage of fetal development at

6.5dpc. Examinations conducted at 7.5dpc, however, revealed striking

15   differences in the numbers and appearances of conceptus. The average

number of allogeneic conceptus + inhibitor was reduced to 4.5 per female at

7.5dpc and declined to <2 at 8.5/9.5dpc. No conceptus were present on any

mice carrying allogeneic conceptus and treated with 1IDO inhibitor after

9.5dpc. Normal numbers of healthy allogeneic (-IDO inhibitor) and

20   syngeneic (±IDO inhibitor) conceptus were present in control groups.

Methods. Mice were mated, treated with IDO inhibitor and dissected at

gestation times indicated. Tissues were prepared for sectioning by fixing

them in 4% paraformaldehyde. Serial sections (5μm) were prepared using a

5   microtome and were stained with hemtoxylin and eosin before microscopic

examination.

On gross examination significant inflammation and excessive

maternal blood surrounded the majority of 7.5dpc allogeneic conceptus in

mice treated with IDO inhibitor. In contrast, maternal blood was localized

10   normally near the ectoplacental cone in all 7.5dpc syngeneic conceptus

treated with IDO inhibitor; their appearance was indistinguishable from that

if syngeneic and allogeneic conceptus dissected from mothers in placebo

groups.

On histological examination increased numbers of enlarged blood

15   vessels within the decidual region were observed in 7.5dpc allogeneic

conceptus when IDO activity was blocked. However, allogeneic 7.5dpc

embryos appeared intact and at the expected developmental stage. One day

later (8.5 dpc) allogeneic conceptus (+IDO inhibitor) were grossly abnormal.

Extensive mixed mononuclear and neutrophil infiltrates were evident in

20   every conceptus and were accompanied by extensive tissue degeneration in

the decidual region. Grossly abnormal embryos were present but

developmentally retarded. At 9.5 dpc no allogeneic embryos were present in

mice treated with IDO inhibitor and decidual swellings were not apparent;

some cellular debris was detected within the uterine lumen.

25   These data reveal that blocking IDO activity during pregnancy has a

profound and specific effect on development of allogeneic conceptus,

leading to loss of all embryos by mid-gestation. Embryo loss was not caused

by surgical manipulations to implant pellets at 4.5 dpc. This data shows that

blocking IDO activity compromises development of conceptus only when

30   there is a genetic difference between mother and embryos. This implies that

functional IDO enzyme activity is essential to protect allogeneic conceptus

during early gestation and that the maternal immune system is involved in

processes that lead to loss of allogeneic conceptus.

5   **Example 10:   Maternal lymphocytes provoke loss of allogeneic**
conceptus when IDO activity is blocked.

The contribution of maternal lymphocytes to experimentally induced

loss of allogeneic conceptus in mice treated with 1-methyl-L-tryptophan was

assessed using females carrying the RAG-1 induced mutation (RAG-1-/-)

10   which prevents development of T or B cells (Li, 1982, Cancer 50, 2066-

2073). As before, female mice carrying disrupted RAG-1 genes (on the

DBA background) were mated with B6 males and treated with 1-methyl-L-

tryptophan (4.5cpd). Pregnancies were continued until 11.5dpc when

females were sacrificed to assess the number of conceptus. Normal numbers

15   of healthy conceptus were found in all females examined (Table 2, first line).

Table 2 -	Maternal lymphocytes and a single MHC I alloantigen induce embryo rejection		
Mating Genotype No.	**IDO**	**No. ♀ examined**	**Mean**
(♀ x ♂)	**Inhibitor**	**(dpc)**	**conceptus/♀**
CBA [RAG-1-/-] x B6	yes	5 (11.5)	7.8
CBA [RAG-1-/-] x B6	yes	2 (20.5, post partum)	7 (pups born)
CBA x GK*	yes	4 (11.5)	0
CBA x GK*	no	21 (11.5)	6.4

*GK mice are H-2K^b-transgenic mice made on the CBA background.

This data demonstrates that maternal lymphocytes are essential

participants in events that precede loss of allogeneic conceptus when IDO

20   activity is blocked. The most likely explanation is that maternal T cells are

responsible for initiating mediating processes that lead to loss of allogeneic

conceptus when IDO activity is blocked. This premise is based on the

assumption that conceptus, like tissue allografts, provoke T cell responses.

Although B cells, via antibody production, can contribute to allograft

25   rejection, it is unlikely that antibodies against fetal alloantigens mediate rapid

loss of conceptus a few days after implantation, especially as virgin females

were used in these experiments.

5   **Example 11:   A single MHC 1 difference provokes loss of allogeneic**
                       conceptus.

         B6 and CBA mice differ at a large number of genetic loci including

the entire MHC region (MHC I and MHC II) as well as at multiple minor

histocompatibility (miHC) loci. To determine whether a single polymorphic

10   MHC molecule could provoke fetal loss, CBA mice were mated with (GK)

transgenic male mice carrying a recombinant H-2K^b (GK) transgene; a

promoter from the human HLA-G gene drives H-2K^b-expression at high

level in murine trophoblast (Zhou, et al. 1998 *J. Reprod. Immunol.* 40, 47-

62). GK mice were made on the inbred CBA strain background and, hence,

15   differ from CBA mice only because of H-2K^b expressed by the GK

transgene. Normal size litters are borne by CBA females after mating with

GK transgenic male mice (Table 2). Pregnant mice were treated with 1-

methyl-L-tryptophan and examined at 11.5dpc, as before, to assess effects on

fetal development (Table 2). No conceptus, nor any resorbed decidua, were

20   observed when mice were examined on 11.5dpc, although, in each mouse,

the uterus was distended indicating that pregnancy had terminated

prematurely.

         This result is important for two reasons. First, a single paternally-

inherited MHC I difference between mother and conceptus is enough to

25   provoke fetal loss when IDO activity is blocked. Second, it eliminates the

possibility that fetal loss occurs after CBA [x B6] matings because of an

inherent genetic predisposition to fetal loss in this mating combination.

         This result supports the view that maternal T cells, not B cells, are

responsible for fetal loss since MHC alloantigens provoke strong T cell

30   responses but weak B cell responses. If true, this implies that maternal CD8$^+$

T cells recognizing native H-2K^b molecules expressed by fetal cells may be

responsible for provoking fetal loss.

         In summary, these data demonstrate that the IDO enzyme inhibitor 1-

methyl-tryptophan has a profound and specific abortifacient effect on all

35   allogeneic conceptus prior to 9.5dpc. Fetal abortion is not due to non-

specific toxic affects of the drug itself as syngeneic conceptus develop

5   normally in females exposed to 1-methyl-tryptophan as do allogeneic

conceptus in the absence of maternal lymphocytes. Alternatively, embryonic

development may be affected by substances produced when 1-methyl-

tryptophan is metabolized by cells expressing IDO. However, this is

unlikely to explain the abortifacient effect of 1-methyl-tryptophan since the

10   IDO gene is expressed in syngeneic and allogeneic conceptus early in

gestation during the period when embryo rejection occurs (7.5-9.5dpc). One

therefore concludes that the abortifacient effect of 1-methyl-tryptophan

arises because it inhibits IDO enzyme activity. Decidual cells expressing

IDO normally establish a protective barrier that prevents maternal T cell

15   mediated rejection of allogeneic conceptus.

Example 12.   Inhibition of Tumor Growth by administration of IDO

inhibitor.

In light of the data herein showing that the allogeneic fetus is able to

protect itself against attack by the maternal immune system through

20   expression of IDO, and given the expression of IDO in tumors as cited

above, it was tested whether a similar mechanism allowed tumor cells to

evade immune attack. The MB49 epithelial carcinoma model was chosen for

proof of concept because it is an aggressive, lethal malignancy when

innoculated into syngeneic hosts, but it can be rendered susceptible to

25   immune attack if the host's immune system is suitably activated (Chen, et al.

1997 *J. Immunol.* 159, 351-359). Tumor-bearing hosts were treated with the

IDO inhibitor 1-methyl-tryptophan. MB49 tumor cells (1×10^6) were

injected subcutaneously into syngeneic C57/B16 hosts. Pellets containing 1-

methyl-tryptophan (0.9 mg/hr, 7-day release) were implanted at the time of

30   tumor cell innoculation. By day 10, all animals had evidence of initial tumor

formation (palpable mass). By day 15, control animals were visibly ill and

the experiment was terminated. Animals were sacrificed on day 11-15 for

histologic examination.

As shown in Table 3, administration of 1-methyl-tryptophan

35   significantly reduced tumor growth in immunocompetent, syngeneic hosts,

compared to vehicle control. Two important points are noted: First, no

5   manipulation of the immune system other than inhibition of IDO was

required for anti-tumor effect – i.e., the tumor itself became immunogenic

once the barrier of IDO was removed. Second, the observed responses

continued at least 4-7 days *after* the period of inhibitor administration (days

1-7), suggesting that an initial priming period was sufficient to generate a

10   sustained anti-tumor immune response.

Table 3. Anti-tumor effects of 1-methyl-tryptophan administration.

Outcome measure	control group (n=7)	1-methyl-tryptophan group (n=7)
complete responses	0	1
partial responses	0	6
size of residual tumor	2 - 2.5 cm	1 cm (necrotic)
histology (day 15)	viable tumor, minor infiltration of CD4+ T cells with occasional CD8+, scattered macrophages	apoptosis, necrosis, and hemorrhage, extensive infiltrate of CD4+ and CD8+ T cells, extensive infiltration of macrophages
visible metastases	extensive local metastases, with gross evidence of intraperitoneal and visceral metastases	none
performance status	decreased activity, failure to groom, ill appearance	normal

15   Modifications and variations of the methods and compositions

described herein will be obvious to those skilled in the art and are intended to

be encompassed by the claims.

We claim:

1.   A method for altering T cell mediated immunity comprising altering local extracellular concentrations of tryptophan and tryptophan metabolites.

2.   The method of claim 1 wherein the extracellular concentration is altered by inhibition or increased activity of IDO mediated metabolism of tryptophan.

3.   The method of claim 1 wherein the tryptophan concentrations are decreased below physiological levels.

4.   The method of claim 1 wherein the tryptophan concentrations are increased above physiological levels.

5.   The method of claim 1 wherein the concentrations are altered to regulate rejection or acceptance of a allograft or xenograft

6.   The method of claim 5 wherein the concentrations of tryptophan are decreased or the concentration of tryptophan metabolites are increased to prevent or terminate pregnancy.

7.   The method of claim 5 wherein the concentrations of tryptophan are increased or the concentration of tryptophan metabolites are decreased to promote pregnancy.

8.   The method of claim 5 wherein the concentrations of tryptophan are increased to decrease the liklihood of rejection of an allograft or xenotransplanted tissue or organ.

9.   The method of claim 1 wherein the concentrations of tryptophan or tryptophan metabolites are altered to induce rejection of cells by a human or animal hosting the cells.

10.   The method of claim 9 wherein the cells are tumor cells.

11.   The method of claim 9 wherein the cells are virally infected or transformed cells.

12.   The method of claim 1 wherein compounds decreasing typtophan degradation are administered in combination with a vaccine to enhance the immune reaction to the vaccine.

13.   The method of claim 1 wherein compounds decreasing typtophan degradation are administered to help reverse the immunosuppressed state

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found in AIDS

14.   The method of claim 1 wherein compounds increasing tryptophan degradation are administered to decrease an autoimmune response.

15.   The method of claim 1 wherein the concentration of tryptophan is altered by administration of a compound inhibiting tryptophan decregation.

16.   The method of claim 1 wherein the concentration of tryptophan is altered by administration of a compound increasing extracellular levels of tryptophan.

17.   The method of claim 1 wherein the concentration of tryptophan is altered locally or topically.

18.   The method of claim 1 wherein the concentration of tryptophan is altered systemically.

19.   The method of claim 1 wherein tryptophan concentration is decreased further comprising administering a cytokine to increase typtophan degradation.

20.   The method of claim 19 wherein the cytokine is MCSF.

21.   The method of claim 19 wherein the composition and cytokine is administered to a patient with an autoimmune disorder.

22.   The method of claim 19 wherein the composition and cytokine is administered to a patient with an allograft or xenograft.

23.   A method for assessing the immunological state of certain cells comprising measuring tryptophan levels, tryptophan metabolite levels or IDO activity.

24.   The method of claim 23 wherein the cells are a tissue biopsy from a patient suspected of having a malignancy.

25.   The method of claim 23 wherein the cells are obtained from a pregnant woman.

26.   A pharmaceutical composition for altering extracellular tryptophan concentrations comprising

         an effective amount of a compound suitable for administration to a patient in need of treatment thereof to alter extracellular levels of tryptophan or typtophan metabolites to modify T cell mediated immunity, and

a pharmaceutically acceptable carrier for administration to a patient.

27.   The composition of claim 26 increasing tryptophan concentrations and decreasing tryptophan metabolite concentrations in extracellular fluid.

28.   The composition of claim 26 decreasing tryptophan concentrations and increasing tryptophan metabolite concentrations in extracellular fluid.

29.   The composition of claim 27 comprising an inhibitor of IDO.

30.   The composition of claim 29 wherein the inhibitor is selected from the group consisting of 1-methyl-DL-tryptophan, β-(3-benzofuranyl)-DL-alanine, β-[3-benzo(b)thienyl]-DL-alanine, and 6-nitro-L-tryptophan).

31.   The composition of claim 28 comprising an enzyme degrading tryptophan.

32.   The composition of claim 31 selected from the group consisting of recombinant IDO and indolyl-3-alkane alpha-hydroxylase.

34.   The composition of claim 26 comprising an inhibitor of the high affinity, sodium independent tryptophan transport system.

35.   The composition of claim 26 in a formulation for controlled or sustained release.

36.   The composition of claim 26 further comprising a vaccine.

37.   The composition of claim 26 in a dosage and formulation for injection into synovial joints to decrease an autoimmune response.

38.   The composition of claim 26 in a dosage and formulation for intravaginal administration for preventing or terminating pregnancy.

FIG. 1

FIG. 2

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FIG. 3

FIG. 4A
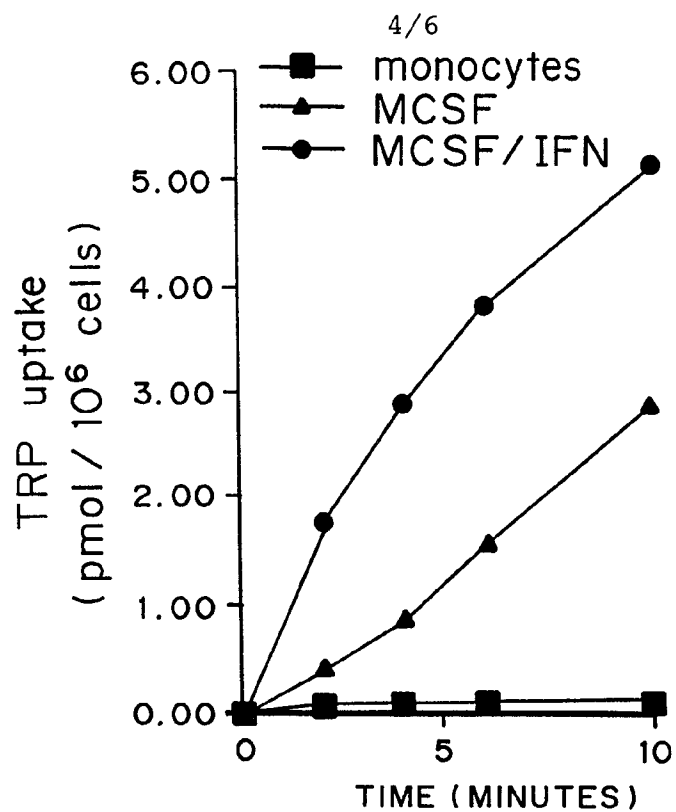Inhibition with
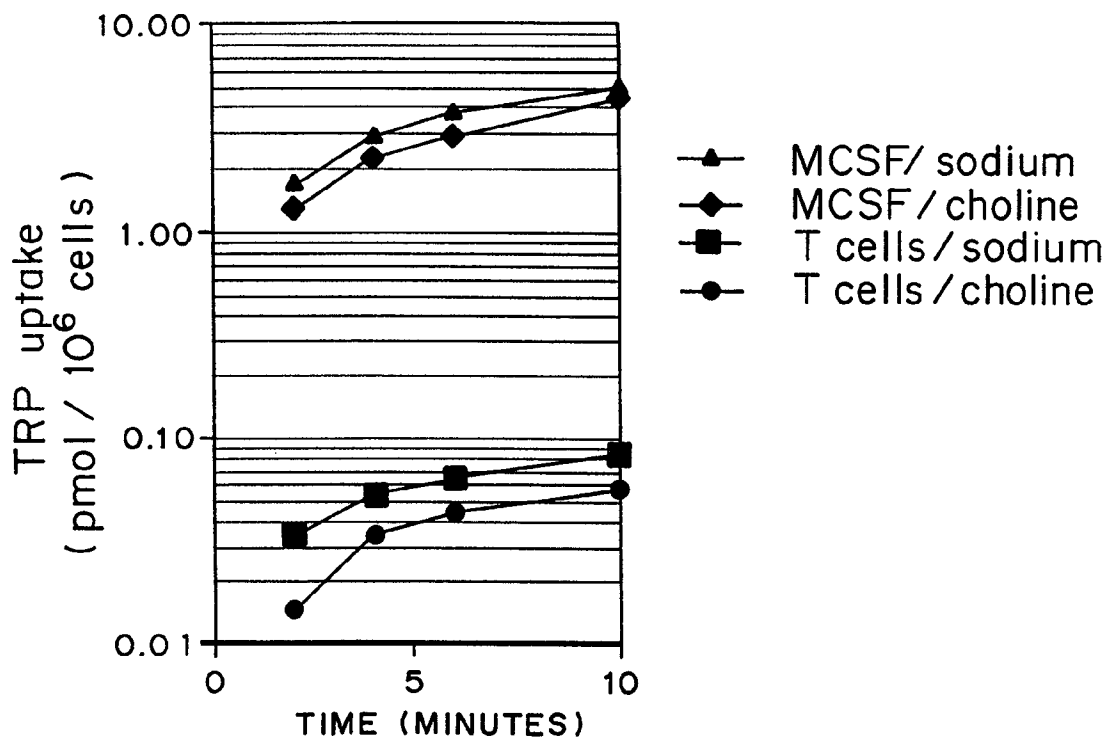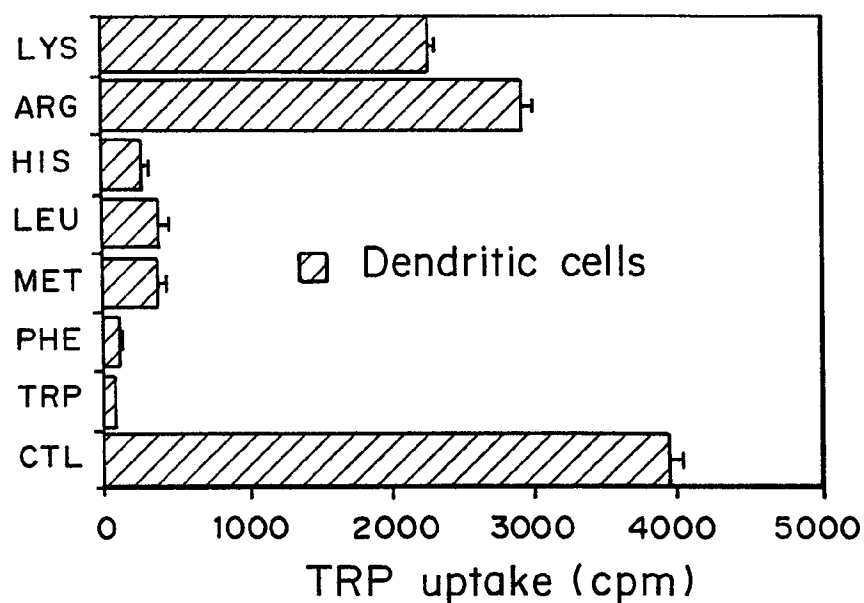1-methyl-TRP

FIG. 4B
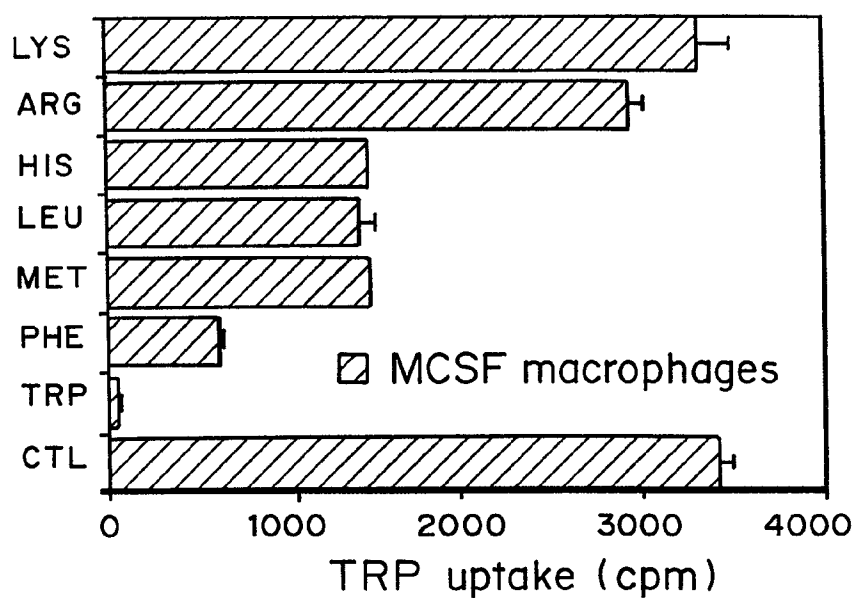Supplementation with
10x TRP

FIG. 5

FIG. 6

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FIG. 7A

FIG. 7B

FIG. 8A

FIG. 8B

FIG. 9

FIG. 10

FIG. 11